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REMARKS/ARGUMENTS

Status of the Claims

Claim 63 has been amended. Claims 63-66 are pending. Support for the amendments to claim 63 may be found throughout the specification. More specifically, support for the phrase "an N-terminal β-lactamase fragment fused to a flexible polypeptide linker and a first interactor domain" may be found, for example, at page 9, lines 31-32 ("an N-terminal fragment fused through a break-point terminus to a flexible polypeptide linker and a first interactor domain"), and at page 5, line 30 to page 6, line 1 ("[t]he fragment complementation system of the present invention involves co-expression in a host cell of a first and a second oligopeptide, where each is a fusion protein separated by a flexible polypeptide linker with a member of the marker protein fragment pair). Support for the phrase "wherein said fragment complementation system is used *in vitro* or in a eukaryotic host cell" may be found, for example, at page 10, line 32, to page 11 line 2 ("[t]he interaction-dependent enzyme activation system of the subject invention may be used to detect *in vitro* protein interactions, such as in cell lysates, or the interactions of intracellular or extracellular proteins of a host cell") and at page 12, lines 6-9 ("[a]ppropriate host cells for application of the subject invention include both eukaryotic cells, such as mammalian, yeast and plant cells, and prokaryotic cells, such as bacterial cells). Therefore, no new matter is added with entry of the present amendments.

35 U.S.C. § 112, First Paragraph: New Matter

The Examiner has rejected claims 63-66 as allegedly containing subject matter not described in the specification in such a way as to reasonably convey to one skilled in the art that Applicants were in possession of the claimed invention. The Examiner asserts that the application as filed does not support "at least one" amino acid substitution selected from K55E, P62S and M182T. Applicants respectfully disagree.

Original claim 41 recites:

> 41. The fragment complementation system according to Claim 35, wherein said N-terminal

fragment comprises at least one mutation selected from the group consisting of K55E, P62S and M182T.

Original claim 35 recites:

> 35. A fragment complementation system, said system comprising:
> a first oligopeptide comprising an N-terminal fragment of a β-lactamase fused through a break-point to a flexible polypeptide linker and a first interactor domain; and
> a second oligopeptide comprising a second interactor domain and a flexible polypeptide linker fused through a break-point to a C-terminal fragment of a β-lactamase, wherein said N-terminal and said C-terminal fragment functionally reconstitute said β-lactamase upon binding of said first interactor domain with said second interactor domain.

One skilled in the art would immediately recognize that claim 41 explicitly recites that the N-terminal fragment mutations K55E, P62S and M182T may be present in the alternative or in combination. Because claim 41 is part of the application as originally filed, Applicants respectfully assert that claim 63 contains no new matter. Therefore, Applicants respectfully request withdraw of the rejection of claims 63-66 under 35 U.S.C. §112, first paragraph as allegedly containing new matter.

35 U.S.C. § 112, First Paragraph: Enablement

The Examiner has rejected claims 63-69 as allegedly nonenabled. The Examiner asserts:

1. Applicants' use of "comprising" language results in overly broad claims encompassing an infinite number of sequences and undisclosed amino acids having an unpredictable effect on the structure and/or function of the N-terminal fragment; and
2. The claims lack the "critical" features of a tripeptide, a linker, and a C-terminal β-lactamase fragment.

In response to the Examiner's rejections, Applicants have removed the "comprising" language from claim 63, included a linker fused to the N-terminal β-lactamase fragment and the first interactor domain, and specified that the claimed oligopeptide "is used *in vitro* or in a eukaryotic host cell." In addition, Applicants herewith submit Galarneau *et al.*, *Nat. Biotech.*, **20**: 619-622 (2002) as Exhibit A. Galarneau *et al.* is a scientific article published after the effective filing date of the present application. Galarneau *et al.* provides evidence that tripeptides are not a critical feature of the claimed oligopeptide. In light of these amendments and the remarks below, Applicants respectfully assert that claims 63-66 as amended are fully enabled.

The test for enablement is whether one of ordinary skill in the art could make or use the invention from the disclosure coupled with information known in the art without undue experimentation. MPEP § 2164.01. The enablement requirement is satisfied when the specification discloses at least one method for making and using the claimed invention that bears a reasonable correlation to the scope of the claims. MPEP § 2164.01(b); *In re Fisher*, 166 USPQ 18, 24 (CCPA 1970). While a feature that is taught as critical must be recited in the claim, Applicants note that:

> In determining whether an unclaimed feature is critical, the entire disclosure must be considered . . . **Broad language** in the disclosure, including the abstract, omitting an allegedly critical feature, **tends to rebut the argument of criticality**.

See MPEP § 2164.08(c) (emphasis added).

Use of "Comprising" Language

The Examiner asserts that the use of "comprising" language in claim 63 results in overly broad claims encompassing an infinite number of sequences and undisclosed amino acids having an unpredictable effect on the structure and/or function of the N-terminal fragment. In response, Applicants have amended claim 63 to recite "[a]n oligopeptide for use in a fragment complementation system ***consisting essentially of*** . . . [emphasis added]." According to the MPEP, "[t]he transitional phrase 'consisting essentially of' limits the scope of a claim to the

specified materials or steps 'and those that do not materially affect the basic and novel characteristic(s)' of the claimed invention [quoting *In re Herz*, 537 F.2d 549 (CCPA 1976)]."

Therefore, Applicants respectfully assert that claim 63 as amended does not encompass an infinite number of sequences and undisclosed amino acids having an unpredictable effect on the structure and/or function of the N-terminal fragment. Rather, claim 63 now encompasses only those sequences and amino acids "that do not materially affect the basic and novel characteristic(s)" of the claimed oligopeptide.

Non-Critical Features

The Examiner asserts that the claims lack the following "critical" features: a tripeptide, a linker, and a C-terminal β-lactamase fragment. Because Applicants have amended the claims to include a linker fused to the N-terminal β-lactamase fragment and the first interactor domain, the issue of whether the linker is a critical feature is rendered moot. Furthermore, Applicants submit that the tripeptide and C-terminal β-lactamase fragment are not critical features of the claimed oligopeptide.

A Tripeptide is Not a Critical Feature of the Claimed Invention

Applicants submit herewith Galarneau *et al.* as Exhibit A. Galarneau *et al.* reports successfully using an oligopeptide in a complementation system. The oligopeptide contains an N-terminal TEM-1 β-lactamase fragment containing amino acids 26-196 of SEQ ID NO:2. See page 620, Figure 1. In addition, complementation is demonstrated using the M182T mutant ***in the absence of a tripeptide***. See page 621, Figure 3. The utility of this complementation system was demonstrated ***in vitro and in eukaryotic cells***. See page 621, Figure 3 ("*In vitro* and *in vivo* protein-protein interactions studies using the TEM-1 β-lactamase PCA").

The Galarneau *et al.* study validates Applicants' teachings in the specification, which repeatedly states that the claimed oligopeptide may ***optionally*** contain tripeptides. For example, in the "Summary" of the invention on page 6, lines 5 to 9, the specification states:

> Functional reconstitution of the fragment pairs into a marker protein ***can be enhanced*** by including elements such as a cysteine residue ***or*** a randomly encoded peptide of from 3-12 amino acids at or near the break-point termini of the fragment pair member, ***or*** by introducing 1-3 codon changes within the nucleotide sequence encoding for a member of a fragment pair (emphasis added).

In this passage, the specification clearly contemplates the use of an oligopeptide containing a "1-3 codon" change (such as M182T) ***or*** a randomly encoded peptide of from 3-12 amino acids (such as a tripeptide). After reading this passage, one of skill in the art would immediately recognize that tripeptides are ***optional*** and not a critical feature of the claimed oligopeptides.

In asserting that tripeptides are a "critical" feature of the claimed invention, the Examiner has cited Example 7 of Applicants' specification, which states:

> Thus, in contrast to the results obtained with random tri-peptides, where activation remained interaction-dependent, adaptive mutations of α197 invariably eliminated interaction dependence.

See page 48, lines 6-9. However, this statement regarding the effect of tripeptides and adaptive mutations on the interaction-dependence of the complementation system were made in reference to the specific conditions set forth in Example 7. In Example 7, the complementation system was tested ***in vivo*** using ***prokaryotic*** DH5 alpha cells (an *E.coli* strain). Similarly, the post-filing date reference cited by the Examiner, Wehrman *et al.*, *Proc. Nat'l. Acad. Sci.*, **99**: 2469-2479 (2002), also tested the complementation system *in vivo* using prokaryotic DH5 alpha cells. See page 3470, column 1, line 13. The fact that adaptive mutations eliminated interaction-dependence of an oligopeptide having a tripeptide in a ***prokaryotic*** cell has no bearing on claim 63 as amended. Claim 63 now specifies that the claimed oligopeptide "is used ***in vitro or in a eukaryotic host cell***."

The Examiner is respectfully reminded that "[i]n determining whether an unclaimed feature is critical, ***the entire disclosure*** must be considered." See MPEP §2164.08(c). Applicants submit that it is improper to focus only on a statement made in reference to tests conducted ***in vivo*** using ***prokaryotic*** cells, while ignoring explicit statements in the specification

clearly contemplating the use of tripeptides as an ***optional*** means of enhancing reconstitution. See page 6, lines 5 to 9.

Moreover, the determination of enablement should always be based on the weight of all the evidence before the Examiner. See MPEP § 2164.05. Here, Applicants have presented Galarneau *et al.* as Exhibit A, which reports successfully using the M182T mutant ***in the absence of a tripeptide*** in a complementation system tested ***in vitro and in eukaryotic cells***.

In sum, Applicants respectfully submit that the tripeptides are not a critical feature of the claimed oligopeptide, as stated in the Applicants' specification and as demonstrated in the Galarneau *et al.* studies.

A C-terminal Fragment is Not a Critical Features of the Claimed Invention

In the Examiner's discussion of allegedly "critical" features of the invention, the Examiner notes that "only complementation systems that contain Asn-Gly-Arg tripeptide (NGR) and the $(Gly_4Ser)_3$ linker in addition to the complimentary C-terminal β-lactamase fragment would be enabled." See page 5, lines 20-22. In as much as the Examiner is asserting that a complimentary C-terminal β-lactamase fragment is a critical feature of the claimed invention, Applicants respectfully disagree.

The Examiner is respectfully reminded that claims 63-66 are drawn to an oligopeptide for use in a fragment complementation system, not the complementation system itself, and not a method of using the oligopeptide. By requiring Applicants to claim another component of the complementation system, the Examiner is essentially denying Applicants the opportunity to claim any single component. If the Examiner's position were proper, inventors would be forbidden from claiming any individual component of a novel system. For example, a claim drawn to a novel carburetor for use in a novel engine would be rejected as failing to recite the essential component of the novel engine. However, the patent code does not deny inventors the right to claim novel components of novel systems. Therefore, Applicants respectfully assert that claims 63-66 as amended are fully enabled without the recitation of a complimentary C-terminal β-lactamase fragment.

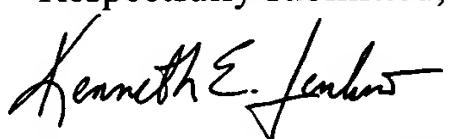
In light of the amendments to the claims and the above remarks, Applicants respectfully request that all the rejections based on 35 U.S.C. § 112, first paragraph be withdrawn.

CONCLUSION

In view of the foregoing, Applicants believe all claims now pending in this Application are in condition for allowance and an action to that end is respectfully requested.

If the Examiner believes a telephone conference would expedite prosecution of this application, please telephone the undersigned at 415-576-0200.

Respectfully submitted,



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β-Lactamase protein fragment complementation assays as *in vivo* and *in vitro* sensors of protein–protein interactions

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We have previously described a strategy for detecting protein–protein interactions based on protein interaction–assisted folding of rationally designed fragments of enzymes. We call this strategy the protein fragment complementation assay (PCA)[1–5]. Here we describe PCAs based on the enzyme TEM-1 β-lactamase (EC: 3.5.2.6), which include simple colorimetric *in vitro* assays using the cephalosporin nitrocefin and assays in intact cells using the fluorescent substrate CCF2/AM (ref. 6). Constitutive protein–protein interactions of the GCN4 leucine zippers and of apoptotic proteins Bcl2 and Bad, and the homodimerization of Smad3, were tested in an *in vitro* assay using cell lysates. With the same *in vitro* assay, we also demonstrate interactions of protein kinase PKB with substrate Bad. The *in vitro* assay is facile and amenable to high-throughput modes of screening with signal-to-background ratios in the range of 10:1 to 250:1, which is superior to other PCAs developed to date. Furthermore, we show that the *in vitro* assay can be used for quantitative analysis of a small molecule–induced protein interaction, the rapamycin-induced interaction of FKBP and yeast FRB (the FKBP-rapamycin binding domain of TOR (target of rapamycin)). The assay reproduces the known dissociation constant and number of sites for this interaction. The combination of *in vitro* colorimetric and *in vivo* fluorescence assays of β-lactamase in mammalian cells suggests a wide variety of sensitive and high-throughput large-scale applications, including *in vitro* protein array analysis of protein–protein or enzyme–protein interactions and *in vivo* applications such as clonal selection for cells expressing interacting protein partners.

For this new PCA design, we used the 29 kDa isoform product of the ampicillin resistance gene (*amp*^{r}) TEM-1 β-lactamase. This isoform lacks the periplasmic secretory signal sequence consisting of the first 23 amino acids[6]. The TEM-1 β-lactamase from *Escherichia coli* meets the essential criteria for a PCA candidate[7,8]. It is relatively small and monomeric, is well characterized structurally and functionally, can be easily expressed, and is not toxic to prokaryotic and eukaryotic cells[9,10]. Furthermore, no orthologs of β-lactamase exist in eukaryotes, and thus a PCA based on β-lactamase could be used universally in eukaryotic cells and many prokaryotes without any intrinsic background activity. β-Lactamase also has the desirable features of enzymatic amplification and facile *in vivo* and *in vitro* assays. Finally, we can take advantage of the versatile fluorescent β-lactamase substrate, CCF2/AM, which allows ratiometric fluorescence detection and thus superior reproducibility and quantification of results in intact cells[6].

The strategy for the selection and design of fragments is described elsewhere[7,8]. On the basis of an analysis of the β-lactamase structure, we proposed to dissect the enzyme between Gly196 and Leu198, because this site is located on a surface opposite to the active site (Fig. 1B) and produces fragments of approximately the same length. It also contains no periodic secondary structure, and it is topologically feasible for the protein to fold. To test whether altering topology in the proposed fragmentation site would affect the folding of the enzyme, we investigated whether increasing the flexibility or producing a circular permutation of the sequence at the Gly196/Leu198 interface would preserve β-lactamase activity (Fig. 2A). Using nitrocefin as substrate, we tested for β-lactamase activity in cell lysates (1:20 dilution) for wild-type, flexible, and circularly permuted forms expressed in COS-7 cells (Fig. 1C, D). The β-lactamase with the flexible linker insert (QI) retained 40% activity, whereas the circular permutant (CP) form retained 20% of wild-type activity *in vitro*. This demonstrates that altered topology of β-lactamase does not importantly affect its activity, consistent with previous studies of a TEM-1 β-lactamase ortholog[11,12].

We generated two fragments of the enzyme, referred to here as BLF[1] and BLF[2] (Fig. 1B, lower panels). We also created a mutant of the first fragment, BLF[1]MT (M182T), that is known to disrupt an inactive molten-globule intermediate of β-lactamase, reasoning that this mutant could be both more active and metabolically more stable[13,14].

To demonstrate the generalizability of the assay we tested several known protein–protein interactions, including homodimerizing GCN4 parallel coiled-coil leucine zipper (ZIP)[8], the heterodimerizing soluble Bad and truncated (Bcl2T) pair, homodimerizing Smad3, and PKB and its substrate Bad (Fig. 3A). Using the *in vitro* nitrocefin assay, we noted that the BLF[1]MT gave significantly superior results compared with the wild-type BLF[1]. Hydrolysis rates indicate that much higher signal levels are achieved (between 10-fold and 250-fold greater than control background levels, normalized to protein content). All interactions were specific as judged by control experiments using noninteracting proteins. An interaction between ZIP and Bcl2T can be explained by the fact that the Bcl-2 family BH3 domain, which binds to Bcl2T, has a consensus sequence very similar to the heptad repeats in the GCN4 leucine zipper[15]. Interactions can also be detected in intact cells using CCF2/AM, even with the wild-type BLF[1] (Fig. 3A–D).

To show that the β-lactamase PCA could be used for quantitative assessment of induced protein–protein interactions, we tested the rapamycin-induced interaction of FKBP and FRB fused to the β-lactamase fragments *in vitro* (Fig. 3E). Nitrocefin assays were conducted as described above, and in all cases, rapamycin, FK506, nitrocefin, and lysates of COS-7 cells expressing only one or the other FKBP– or FRB–β-lactamase fragment fusion were added to the reaction mix simultaneously. Results were consistent with the known pharmacologic response, with single-site saturable binding and a calculated K_d of 5 nM. The binding was also found to be specific, as inferred from the lack of interaction with ZIP, and FK506 competition for rapamycin binding to FKBP, with a K_i of 450 nM (Fig. 3F)[2,16].

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**Corresponding author (stephen.michnick@umontreal.ca).*

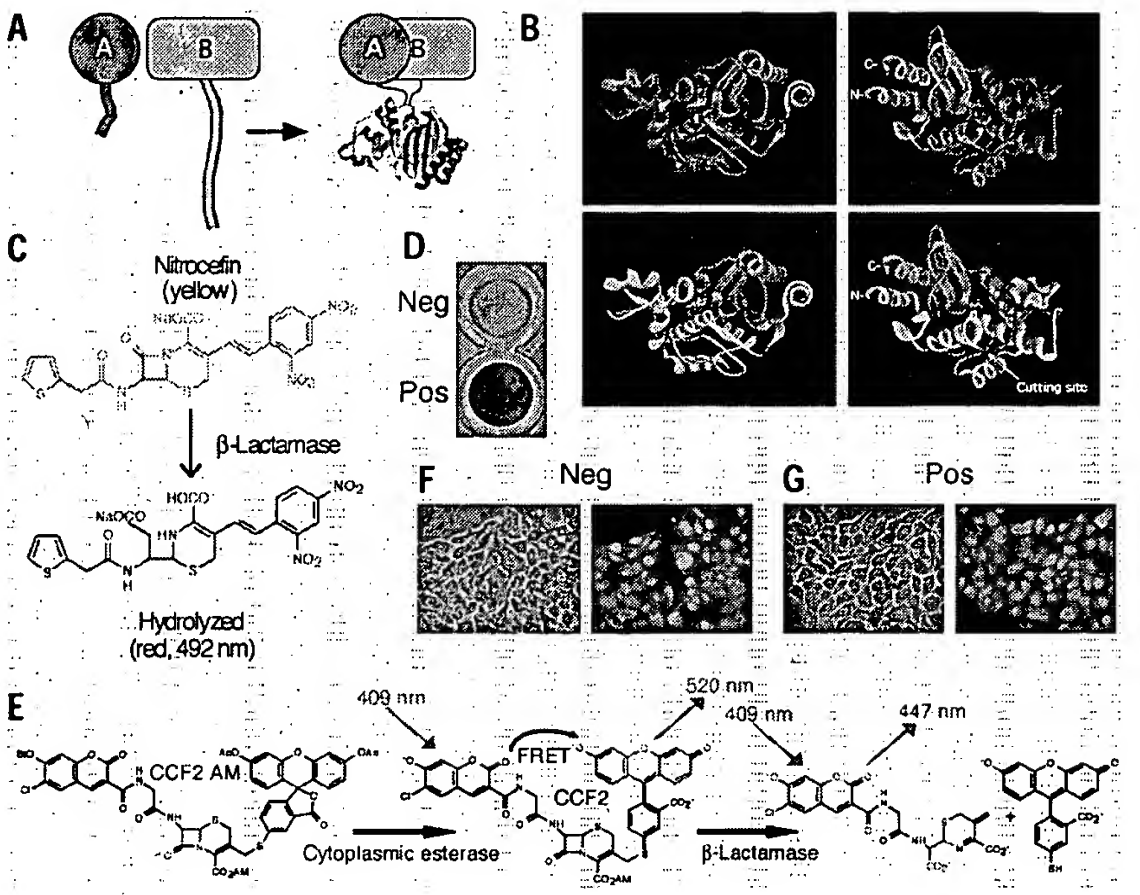

Figure 1. The β-lactamase PCA strategy. (A) Schematic representation of the PCA strategy used to study protein–protein interaction. Interaction between proteins A and B fused to fragments of β-lactamase bring fragments into proximity, allowing for correct folding and reconstitution of enzyme activity. (B) TEM β-lactamase structure[20] illustrating the fragmentation site rendered in Weblab software (Molecular Simulations Inc., San Diego, CA). Upper panels: illustration of the two domains of β-lactamase. The α/β-domain consists of the first 40 and the last 75 amino acids on the sequence (pink). The second, α-domain consists of the 148 amino acids flanked by the sequences that constitute the α/β-domain (blue). Lower panels: the two fragments generated to create a working β-lactamase PCA. The β-lactamase fragments consist of amino acids 26–196 (BLF[1] in cyan) and 198–290 (BLF[2] in red), respectively. A transition state–intermediate analog [[*N*-(benzyloxycarbonyl)amino] methyl]phosphate (green) is shown, surrounded by active site residues (yellow). (C, E) Schematic representations of the *in vitro* and *in vivo* assays, respectively, using substrates hydrolyzed by the β-lactamase enzyme. The *in vitro* assay (C) uses the chromogenic substrate nitrocefin that changes from yellow to red when hydrolyzed by β-lactamase (D). Hydrolysis rates are determined by increasing absorbance at 492 nm. The *in vivo* assay uses CCF2/AM (E) (adapted from ref. 6). When no β-lactamase activity is present, excitation of the coumarin at 409 nm leads to FRET to the fluorescein acceptor and emission of green fluorescence at 520 nm as shown in (F), where HEK 293 cells were transfected with pcDNA3.1 expression plasmid containing no insert. When β-lactamase catalyzes the opening of the β-lactam ring (red, in E), the fluorescein is eliminated and FRET no longer occurs. The coumarin then emits blue fluorescence at 447 nm as seen in (G), HEK 293 cells transfected with wild-type β-lactamase. Nontransfected cells (green) show no evidence of substrate hydrolysis (G).

The TEM-1 β-lactamase PCA described here allows the detection of protein–protein interactions with no apparent background due to spontaneous folding of the enzyme from its fragments. Also, the reporter enzyme is monomeric, thus assuring that interactions will be unambiguously binary. Both *in vivo* and *in vitro* assays are facile and inexpensive to do, and require no specialized equipment. Among alternative experimental strategies, fluorescence resonance energy transfer (FRET) strategies can provide the same information, but their requirement of careful matching of expression levels for fluorochrome-tagged proteins prevents efficient use in large-scale applications[17]. The β-galactosidase subunit complementation strategy suffers from background interference due to spontaneous subunit assembly, and complex subunit assembly results in ambiguous interpretation of stoichiometry[18]. The simplicity, sensitivity, and robustness of the *in vitro* β-lactamase PCA assay may prove useful for large-scale analysis of protein–protein interactions. Finally, the CCF2/AM-based assay could be applied to positive and negative selection for interactions between expressed genes in the same manner as selection for single-gene expression has been demonstrated both in individual cells and in whole organisms[19].

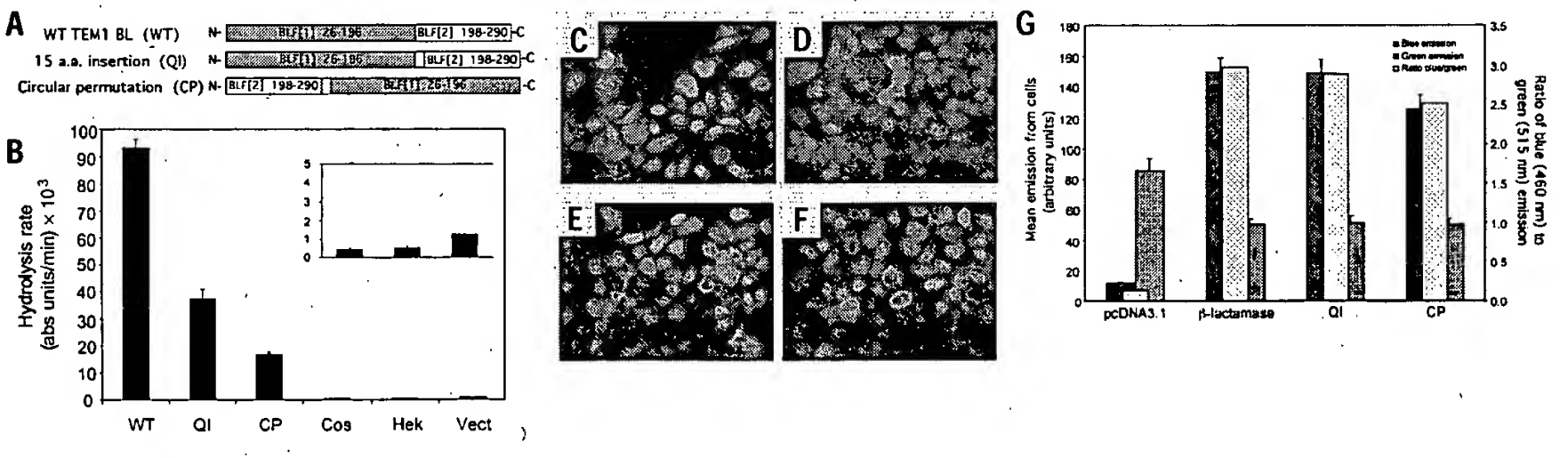

Figure 2. Validation of the chosen fragmentation site. (A) Schematic representation of the quintapeptide insertion (QI) and the circular permutation (CP) in comparison with the wild-type (WT) TEM-1 β-lactamase. (B) *In vitro* assay using nitrocefin. Hydrolysis rate determined from lysates (diluted 1:20) of COS-7 cells transiently transfected with the corresponding fusions shown in (A). Background from nontransfected COS-7 and HEK 293 cells or from mock pcDNA3.1–transfected COS-7 cells is also shown; the upper right histogram shows these negative control results with the *y*-axis expanded 20×. Mean hydrolysis rates were determined for three independent samples and normalized for cell number and protein content. (C–F) Fluorescence microscopy of HEK 293 cells transiently expressing mock pcDNA3.1 (C), wild-type TEM-1 β-lactamase (D), the quintapeptide insertion fusion (E), or the circularly permuted enzyme (F). The transfection efficiencies were between 70% and 85% on the basis of the ratio of blue to green cells in at least 20 fields of view. (G) Image analysis of panels (C–F) with absolute intensities and ratios of blue (460 nm) to green (515 nm) fluorescence in the fields of view represented.

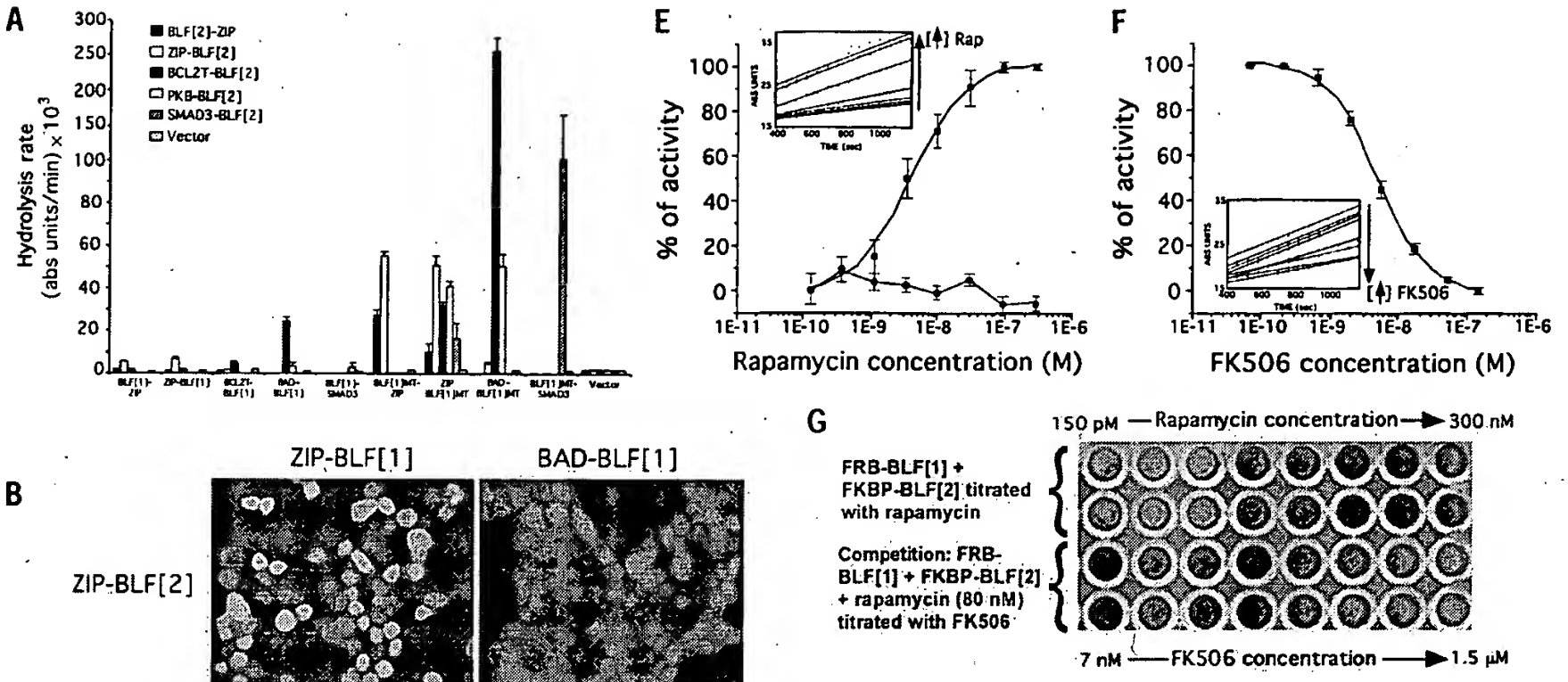

Figure 3. *In vitro* and *in vivo* protein–protein interaction studies using the TEM-1 β-lactamase PCA. (A) *In vitro* hydrolysis of nitrocefin for different interacting pairs of proteins using the wild-type BLF[1] or the M182T mutant BLF[1]MT. Positive interactions include the homodimer of GCN4 leucine zippers (ZIP + ZIP), the anti-apoptotic homodimer of Bcl2 (Bcl2T + Bcl2T), and the heterodimeric pro-apoptotic Bad with anti-apoptotic Bcl2 (Bad + Bcl2T). In addition, the homodimerization of Smad3 was tested. Mean hydrolysis rates were determined for three independent samples and normalized for cell number and protein content. (B) *In vivo* microscopy study. HEK 293 cells were transiently cotransfected with the corresponding fusions. Positive interactions include the GCN4 leucine zippers and Bad with Bcl2T. Negative controls include "noninteracting" pairs (zippers with Bad or Bcl2T). (C) *In vivo* analysis of total HEK 293 cells expressing the corresponding fusion. Cells (1×10^6) were analyzed by plate reader for blue (458 nm) and green fluorescence (520 nm). Mean ratio (blue/green) was determined for three independent samples. Rfu, relative fluorescence units. (D) Cells tested for β-lactamase activity in (C) were collected, lysed, and tested for protein expression of Bad and Bcl2T in fusion with corresponding fragment by western blot. (E) Dose–response curve for rapamycin based on the rate of hydrolysis of nitrocefin by lysates of COS-7 cells transiently coexpressing FRB-BLF[1] and FKBP-BLF[2] (■) or FRB-BLF[1] and ZIP-BLF[2] (●). Mean hydrolysis rates were determined for three independent samples at each rapamycin concentration (inset, raw data showing increase in hydrolysis rates with increase of rapamycin concentration). (F) Competition curve with the inhibitor FK506, an analog of rapamycin. Mean hydrolysis rates were determined for three independent samples at each inhibitor concentration (corresponding to a ratio of rapamycin/FK506 of 1:0 to 1:250). The concentration of rapamycin was kept constant at 80 nM (inset, raw data showing decrease in slope with increase of FK506 concentration). (G) Representative images of microtiter plate results used to construct the dose–response curve shown in (E) and (F).

Experimental protocol

DNA constructs. The vector pQE32, harboring the β-lactamase coding sequence, was used as a template for PCR generation of β-lactamase with features allowing subcloning and separate expression of BLF[1] and BLF[2] (as defined in Results). Complementary oligonucleotides containing new restriction sites, including *Kpn*I, *Hin*dIII, *Not*I, and *Xho*I, were hybridized together and ligated to pcDNA3.1/Zeo linearized with *Kpn*I/*Xho*I. Complementary oligonucleotides containing restriction site *Hin*dIII/*Not*I and coding in frame for a 15-amino-acid flexible polypeptide linker consisting of $(GGGGS)_3$, were hybridized together and ligated into pcDNA3.1/Zeo linearized with *Hin*dIII/*Not*I. The PCR-generated products of BLF[1] and BLF[2] were inserted upstream or downstream, and in frame with the 15-amino-acid linker, with *Kpn*I/*Hin*dIII and *Not*I/*Xho*I, respectively. This led to the creation of the insertion construct BLF[1]-15aa-BLF[2] and the circularly permuted construct BLF[2]-15aa-BLF[1], respectively. Interacting protein-coding sequences generated by PCR, containing either *Kpn*I/*Hin*dIII or *Not*I/*Xho*I, were ligated respectively upstream or downstream of the 15-amino-acid linker, with the exception of Smad3 clones, which were inserted via *Asc*I/*Xho*I. Site-directed mutagenesis was carried out according to the QuickChange method (Stratagene, Cedar Creek, TX), except that we used Platinum Pfx DNA polymerase (Life Technologies, Grand Island, NY). All constructs were confirmed by DNA sequencing.

Western blot analysis. Cells were collected and lysed with a single-detergent buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 100 µg/ml phenylmethylsulfonyl fluoride, 1 µg/ml aprotinin, 0.1% SDS) for 5 min on ice. Approximately 10 µg of total cell extract (based on Bradford assay) was separated by SDS–PAGE and analyzed with p-Bad136 and Bcl2 (C-2) antibodies.

β-Lactamase PCA colorimetric assay. COS-7 or HEK 293 cells were split 24 h before transfection at 1×10^5 in 12-well plates (Corning Star, Acton, MA) in DMEM (Life Technologies) enriched with 10% (vol/vol) Cosmic calf serum (Hyclone, Logan, UT). Cells were transiently cotransfected with 1 µg plasmid DNA using Fugene 6 (Roche Diagnostics, Laval, QC, Canada). Forty-eight hours after transfection, 5×10^6 cells were washed twice with PBS (Life Technologies) and resuspended in 100 µl of 100 mM phosphate buffer, pH 7.0, then lysed by three freeze–thaw cycles (freezing in dry ice/ethanol for 10 min and thawing in a waterbath at 37°C for 10 min). Cell membrane and debris were removed by centrifugation at 16,000*g* for 2 min at 4°C. Assays were conducted in 96-well microtiter plates (Corning Costar). To test β-lactamase activity, 100 µl of phosphate buffer (100 mM, pH 7.0) was added to each well to a final

concentration of 60 mM, containing 2 µl of 10 mM nitrocefin (final concentration 100 µM; Becton Dickinson Microbiology Systems, Cockeysville, MD) and 20 µl of cell lysate, and diluted up to 200 µl with deionized water. Assays were done on a Perkin-Elmer HTS 7000 Series Bio Assay Plate Reader (Montreal, QC, Canada) fitted with a 492 nm filter in the absorption mode. Hydrolysis rates were calculated from plots of the linear range of increasing absorbance at 492 nm, monitored over 20 min. Data were normalized against lysate protein content determined by a Bradford assay (Bio-Rad, Mississauga, ON, Canada).

β-Lactamase PCA fluorogenic assays. HEK 293 cells were split 24 h before transfection at 1.8×10^5 cells onto 15 mm glass coverslips for microscopy (Ted Pella, Reading, PA) in six-well tissue culture plates (Corning Star) in DMEM (Life Technologies) enriched with 10% Cosmic calf serum (Hyclone). Cells were transiently cotransfected using Fugene 6 transfection reagent according to the manufacturer's instructions (Roche Diagnostics). Forty-eight hours after transfection, cells were washed twice with PBS and once with a physiologic saline buffer (10 mM HEPES, 6 mM sucrose, 10 mM glucose, 140 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, pH 7.35) before being loaded for one hour at room temperature with 1.5 µM CCF2/AM. Cells were washed twice with the physiologic saline buffer. For microscopy, cell fluorescence was observed by excitation of CCF2 through a 405 nm filter (20 nm bandpass) with emission observed at 460 nm (50 nm bandpass; blue fluorescence) or 515 nm (20 nm bandpass; green fluorescence). Fluorescence microscopy was conducted on live HEK 293 cells with a Nikon Eclipse (Nikon Canada, Montreal, QC, Canada) TE-200 inverted microscope and a 40× plan fluor dry objective with a numeric aperture of 0.75. Images were taken with a cooled (–50°C) digital charge-coupled device camera (model Orca-II; Hamamatsu Photonics, Bridgewater, NY). Fluorescence spectroscopy was done on samples of 1×10^6 cells mechanically suspended in the physiologic saline buffer, using a Gemini XS (Molecular Devices, Sunnyvale, CA) with excitation at 400 nm and emission at 458 nm (blue) and 520 nm (green).

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The amplicon-plus system for high-level expression of transgenes in plants

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Many biotechnological applications require high-level expression of transgenes in plants. One strategy to achieve this goal was the production of potato virus X (PVX) "amplicon" lines: transgenic lines that encode a replicating RNA virus vector carrying a gene of interest[1]. The idea was that transcription of the amplicon transgene would initiate viral RNA replication and gene expression, resulting in very high levels of the gene product of interest. This approach failed, however, because every amplicon transgene, in both tobacco and *Arabidopsis thaliana*, was subject to post-transcriptional gene silencing (PTGS)[1–3]. In PTGS, the transgene is transcribed but the transcripts fail to accumulate as a result of sequence-specific targeting and destruction[4,5]. Even though the amplicon locus is silenced, the level of β-glucuronidase (GUS) activity in a PVX/GUS line is similar to that in some transgenic lines expressing GUS from a conventional (not silenced) GUS locus[1]. This result suggested that the very high levels of expression originally envisioned for amplicons could be achieved if PTGS could be overcome and if the resulting plants did not suffer from severe viral disease. Here we report that high-level transgene expression can be achieved by pairing the amplicon approach with the use of a viral suppressor of PTGS, tobacco etch virus (TEV) helper component–proteinase (HC-Pro). Leaves of mature tobacco plants co-expressing HC-Pro and a PVX/GUS amplicon accumulate GUS to ~3% of total protein. Moreover, high-level expression occurs without viral symptoms and, when HC-Pro is expressed from a mutant transgene, without detrimental developmental phenotypes.

HC-Pro was an obvious choice for an attempt to rescue the PVX amplicon strategy for high-level expression of transgenes because it suppresses PTGS induced by viruses and by sense transgenes[6–8]. There were a number of reasons, however, that the approach might

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